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Folate reference interval estimation in the Dutch general population

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ABSTRACT

Background: Folate functions as an enzyme co-factor within the one-carbon metabolic pathway, providing key metabolites required for DNA synthesis and methylation. Hence, insufficient intake of folate can negatively affect health. As correct interpretation of folate status is dependent on a well-established reference interval, we set out to perform a new estimation following the restandardization of the Roche folate assay against the international folate standard.

Materials and methods: The folate reference interval was estimated using samples obtained from the Dutch population-based Lifelines cohort. The reference interval was estimated using two methods: a nonparametric estimation combined with bootstrap resampling and by fitting the data to a gamma distribution. The lower reference limit was verified in a patient cohort by combined measurement of folate and homocysteine.

Results: Dependent on the method used for estimation and in- or exclusion of individuals younger than 21 years of age, the lower reference limit ranged from 6.8 to 7.3 nmol/L and the upper reference limit ranged from 26 to 38.5 nmol/L. Applying a lower reference limit of 7.3 nmol/L resulted in the following percentage of folate deficiencies over a period of 12 months: general practitioner 15.5% (IQR 4.0%), general hospital 12.8% (IQR 5.3%), academic hospital 9.6% (IQR 4.3%).

Conclusions: We estimated the folate reference interval in the Dutch general population which is not affected by a folic acid fortification program and verified the obtained lower reference limit by homocysteine measurements. Based on our results, we propose a folate reference interval independent of age of 7.3–38.5 nmol/L.

1. Introduction

Folic acid and its conjugated base folate (vitamin B9 or B11) naturally occur in a wide range of foods and belong to the group of compounds called pterines and B complex vitamins. Folic acid (or folate) is metabolized in the gut and the liver to tetrahydrofolate and

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its biologically active successors (folinic acid amongst others). Supplemental folic acid is fully oxidized and more stable than naturally occurring folic acid. Together with riboflavin, pyridoxal 5'-phosphate and cobalamin (vitamin B2, B6 and B12 respectively), folates act as enzyme co-factors of the cellular one-carbon metabolism. In certain conditions, like intestinal malabsorption or malnutrition, obtaining an adequate level of folates can be challenging resulting in perturbation of one-carbon metabolism. Longstanding perturbation due to folate or cobalamin depletion can result in the development of megaloblastic anaemia, neurological symptoms and DNA damage due to increased incorporation of uracil into genomic DNA [1]. A reduced availability of the active 5-methyltetrahydrofolate and vitamin B12, also results in inadequate remethylation of homocysteine (Hcy), a sulfur-containing non-protein coding amino acid [2]. Hyperhomocysteinemia has been associated with increased risks of coronary and vascular disease, Alzheimer's disease and carcinogenesis [3,4]. Thus, folate diagnostic testing serves a role in the evaluation of several medical conditions, requiring a well-established reference interval (RI) for correct interpretation of test results. In 2016, Roche Diagnostics announced the recalibration of their folate assay against the WHO international folate standard requiring a new estimation of the RI. However, as several countries have implemented folic acid fortification programs to prevent neural tube defects, not every population is suitable to estimate a reliable RI. We determined the RI in the Dutch general population which is not affected by folic acid fortification. We verified the estimated lower reference limit (LRL) by parallel measurement of folate and Hcy in samples from a patient cohort.

2. Materials and methods

2.1. Population and samples

Samples used for the determination of the folate RI were derived from the population-based Lifelines cohort which consists of subjects from the general population living in the northern region of the Netherlands [5]. Cross sectional sampling was performed in July 2016 (186 samples) and February 2017 (220 samples). The samples contained information on sex, age, haemoglobin (Hb) concentration, red blood cell mean corpuscular volume (MCV) and kidney function. Samples met the eligibility criteria when both haematologic parameters were within the sex and age-specific RI's (Male: >18 years Hb 8.5–11 mmol/L/MCV 80–100 fL; 12–18 years Hb 8.3–10.7 mmol/L/MCV 76.7–89.2 fL; 6–12 years Hb 7.3–9.1 mmol/L/MCV 74.4–86.1 fL. Female: >18 years Hb 7.5–10 mmol/L/MCV 80–100 fL; 12–18 years Hb 7.0–9.2 mmol/L/MCV 76.9–90.6 fL; 6–12 years Hb 7.2–8.9 mmol/L/MCV 75.9–87.6 fL) and kidney function was normal (eGFR >60 ml/min/1.73 m²).

For combined analysis of folate and Hcy, samples were derived from leftover patient samples for which analysis of Hcy was ordered. From these samples the age and sex of the patients were recorded and further information discarded. Hcy samples from the department of metabolic diseases were excluded due to possible defects in the one-carbon metabolic pathway which could interfere with the effect of low folate content on Hcy generation. All samples were analysed within 8 hours after collection in EDTA (Hb, MCV) or heparinized tubes (folate, Hcy). Plasma was stored at –20 °C when analysis was not achievable within the same day the sample was collected.

2.2. Folate analysis

Folate was measured in heparinized plasma samples using a competitive binding assay on a Roche Cobas e602 module (Roche Diagnostics, Almere, the Netherlands). Samples used in this study had a hemolysis index $\leq 3 \mu\text{mol/L}$ (hemoglobin $\leq 4.8 \text{ mg/dL}$). The Roche Folate III assay makes use of a ruthenium labelled folate binding protein and biotin labelled folate as tracer. The Folate III assay (Roche reference number 07559992) has been calibrated against the WHO international folate standard (National Institute for Biological Standards and Control code 03/178). Samples above the measuring range ($>45.4 \text{ nmol/L}$ ($>20 \mu\text{g/L}$)) were excluded from the analysis. Folate inter-assay variation: 9.6% at 5.5 nmol/L; 4.7% at 29.1 nmol/L. Folate concentration is expressed both in nmol/L and $\mu\text{g/L}$ (conversion factor 2.266).

2.3. Homocysteine analysis

Plasma total Hcy was measured in heparinized plasma samples using a chemiluminescent microparticle immunoassay (Abbott Diagnostics, Hoofddorp, the Netherlands) with the following inter-assay variation: 5.5% at 7.0 $\mu\text{mol/L}$; 4.9% at 12.4 $\mu\text{mol/L}$; 4.2% at 24.3 $\mu\text{mol/L}$.

Blood samples were collected on melting ice to prevent the formation of Hcy *in vitro*. Samples were stored post-analysis at –20 °C for a maximum of 30 days. Within this period, samples were analysed for folate content.

2.4. Data analysis and statistics

Data analysis was performed using Graphpad Prism 6 (GraphPad Software, California, USA). Comparisons between groups were performed using the two-tailed Mann-Whitney *U* nonparametric test or the Kruskal-Wallis test. Groups with data following a normal distribution were analysed with one-way ANOVA. *P* values < 0.05 were considered significant. The RI (2.5–97.5th percentile) was estimated using nonparametric estimation combined with bootstrap resampling (Analyse-it Software Ltd., Leeds, United Kingdom). In addition, the RI was estimated by fitting data to a gamma distribution. To this aim, differences between fit and data were quadrated to optimize fitting for the peak present in the data. Parameters of the gamma distribution (alpha and beta) were determined by minimizing the difference between fit and data by iteration to convergence (difference < 0.000001). Folate data, used to determine the percentage of results below the lower folate reference limit in different patient populations, were extracted from the laboratory information systems

(LIS) running at the Clinical Chemistry departments of Isala general hospital (Zwolle, the Netherlands) and the University Medical Center Groningen (Groningen, the Netherlands).

3. Results

3.1. Subjects characteristics

Estimation of the folate RI was performed using a cross sectional study in which plasma samples from apparently healthy subjects were measured in a cohort from July 2016 (summer) and a cohort from February 2017 (winter) (Table 1). In the female participants only a significant difference in Hb was seen between both groups. In the male participants mean age, MCV and folate levels were significantly different between both groups.

The combined measurement of Hcy and folate was performed in a patient population consisting of 39 males with a mean age of 27.6 years (95%CI 20.4; 34.8) and 78 females with a mean age of 27.5 years (95%CI 23.3; 31.7).

3.2. Folate reference interval estimation

The folate RI was estimated by measuring a total of 406 plasma samples. The folate dataset was skewed ($\kappa = 1.3$) and did not follow a Gaussian distribution (Shapiro-Wilk test p -value < 0.0001) (Fig. 1a). Separation of the cohort into age groups identified age dependent differences in folate concentration. The 5–20 age group had the lowest median folate concentration (11 nmol/L (4.9 μ g/L)) and differed significantly from all other three age groups (p -value < 0.02 ; Fig. 1b). As age-dependent differences in folate levels have been described before, we decided to estimate the RI's for the combined age groups as well as for the dataset from which the 5–20 age group was omitted.

A Box-Cox transformation [6] ($\lambda = -0.18$) was applied to the complete dataset which decreased the skewness ($\kappa = 0.01$) and resulted in a near normal distribution of the data (Shapiro-Wilk test p -value 0.0585). As normality was not obtained, parametric estimation was not performed. In line with the NCCLS guideline (EP28A) describing the estimation of RI's, a simple nonparametric estimation was used based on ranking data on value [7]. This method was combined with bootstrap resampling to increase precision of the nonparametric estimation [8]. In addition, data were fitted to a gamma distribution (Fig. 1a) to identify possible interference of outliers. This resulted in a comparable LRL but a lower upper reference limit (URL) (Table 2).

Adjusting the dataset by excluding the 5–20 age group ($n = 59$) decreased the skewness slightly but did not change the distribution characteristics ($\kappa = 1.2$, Shapiro-Wilk test for normality p -value < 0.0001). The nonparametric estimation with bootstrap resampling was used to estimate the RI (Table 3). As expected, the LRL was higher compared to the complete dataset with a comparable URL. We decided to use this RI for the whole population, irrespective of age. A gamma distribution fit resulted in a comparable LRL found with the nonparametric method and an URL equal to that identified with the complete dataset.

3.3. Verification of the folate LRL

Reduced folate status has a direct effect on Hcy concentration through reduced availability of the folate molecule 5-methylTHF. An elevated plasma total Hcy concentration is generally defined as $\geq 15 \mu\text{mol/L}$ [9,10]. Nonlinear regression analysis indicated an increase in Hcy levels when folate levels dropped below 10 nmol/L (4.4 μ g/L) (Fig. 2a). Considering the estimated LRL of 7.3 nmol/L (3.2 μ g/L), 48% of samples with folate levels below this LRL had homocysteine concentrations $>15 \mu\text{mol/L}$.

3.4. Folate deficiency in different patient populations

To assess how the use of the new RI would translate in folate deficiencies we analysed the percentage of folate results below the LRL

Table 1
Subjects characteristics.

	Summer		Winter		<i>p</i> -value
	mean	95%CI	mean	95%CI	
Female (n)	99		115		
Age (years)	46.6	43.2; 49.7	44.3	41.2; 47.4	0.35
Hb (mmol/L)	8.6	8.4; 8.7	8.4	8.3; 8.5	0.04
MCV (fL)	89.9	89.3; 90.6	89.6	88.9; 90.3	0.44
Folate (nmol/L)	16.7	15.0; 18.3	18	16.3; 19.6	0.28
Folate (μ g/L)	7.3	6.6; 8.1	7.9	7.2; 8.6	
Male (n)	87		105		
Age (years)	40.7	36.7; 44.7	47.3	44; 50.7	0.013
Hb (mmol/L)	9.3	9.2; 9.5	9.4	9.3; 9.5	0.42
MCV (fL)	88.1	87.3; 88.9	89.4	88.6; 90.2	0.02
Folate (nmol/L)	14.7	13.4; 16	17.7	16.1; 19.3	0.005
Folate (μ g/L)	6.5	5.9; 7.1	7.8	7.1; 8.5	

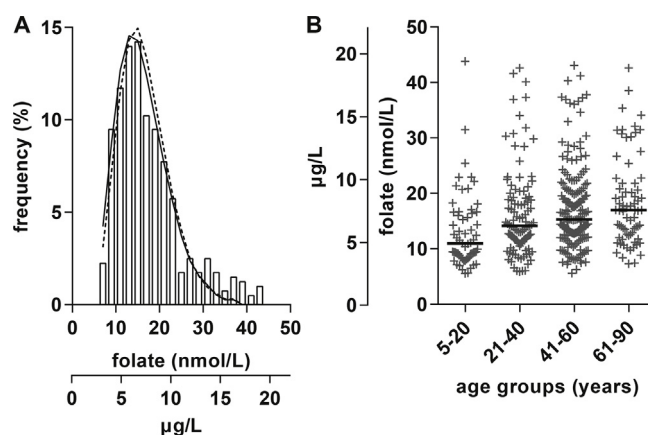


Fig. 1. Folate data characteristics. (A) Frequency distribution of 406 folate measurements. Folate is shown in both nmol/L and corresponding µg/L units. The gamma distribution for the complete dataset and for the dataset excluding the 5–20 age group is depicted as a solid line and dashed line respectively. (B) Data sorted into four age groups (5–20 years $n = 59$; 21–40 years $n = 100$; 41–60 years $n = 173$; 61–90 years $n = 73$). The horizontal bars indicate the median folate concentration within each age group. The 5–20 age group differed from the other age groups (p -value < 0.02 ; Kruskal-Wallis test).

Table 2

Folate RI estimation with the complete dataset.

$n = 406$; $\bar{X} = 16.8$ nmol/L; $s = 7.89$; $k = 1.3$		
nonparametric (bootstrap value 500)		
	Lower limit (90% CI)	Upper limit (90% CI)
nmol/L	6.9 (6.1; 7.4)	38.3 (36.6; 41.1)
µg/L	3.0 (2.7; 3.3)	16.9 (16.1; 18.1)
gamma distribution		
	Lower limit	Upper limit
nmol/L	6.8	26.0
µg/L	3.0	11.5

Table 3

Folate RI estimation with the adjusted dataset.

$n = 347$; $\bar{X} = 17.4$ nmol/L; $s = 7.89$; $k = 1.2$		
nonparametric (bootstrap value 500)		
	Lower limit (90% CI)	Upper limit (90% CI)
nmol/L	7.3 (6.3; 7.8)	38.5 (37.0; 41.2)
µg/L	3.2 (2.8; 3.4)	17.0 (16.3; 18.2)
gamma distribution		
	Lower limit	Upper limit
nmol/L	7.4	26.0
µg/L	3.3	11.5

(7.3 nmol/L (3.2 µg/L)) in a broad range of patients. We expected more folate deficiencies to occur in a general practice setting compared to patient groups requiring a higher level of care. One year of folate data was extracted from two hospital LISs and analysed per month. When the data were sorted based on the healthcare setting (general practitioner (GP), general hospital (GH), academic hospital (AH)), a statistically significant difference between group mean percentages of folate results below the LRL was observed: GP 15.5% (IQR 4.0%), GH 12.8% (IQR 5.3%) and AH 9.6% (IQR 4.3%) (Fig. 2b).

4. Discussion

Estimation of a reliable RI for vitamins can be a challenge due to the immediate influence of food intake or supplement use on vitamin concentrations. In the case of folate, estimation of the RI is further complicated by folic acid fortification programs introduced

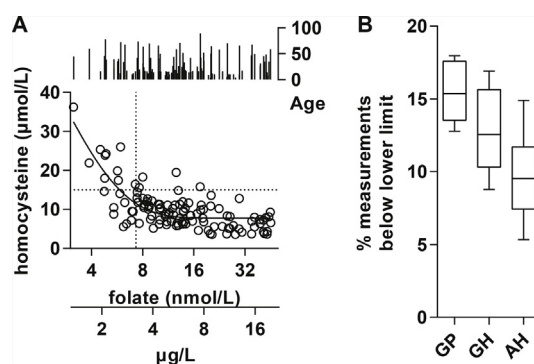


Fig. 2. Verification of the folate lower reference limit. (A) Folate and Hcy measurements from 117 individual patient samples. The vertical dashed line indicates the folate LRL of 7.3 nmol/L (3.2 μg/L), the horizontal dashed line is positioned at 15 μmol/L homocysteine. (B) Percentage of folate measurements below the folate LRL (7.3 nmol/L (3.2 μg/L)) ordered in a 12 month period by general practitioners (GP) $n = 6900$, a general hospital (GH) $n = 6195$ and an academic hospital (AH) $n = 10276$ (one-way ANOVA p -value < 0.0001). Data from separate months are depicted as box plots per clinical setting (one-way ANOVA: GP vs. GH p -value 0.04; GP vs. AH p -value < 0.0001 ; GH vs. AH p -value 0.01).

by several countries which is likely to result in a right shift of the estimated RI. As an example, US data collected during 2011–2012 showed a high mean serum folate concentration of 47.2 nmol/L (20.8 μg/L) with only 0.02% of all samples below 7 nmol/L (3.1 μg/L), measured by HPLC–tandem mass spectrometry [11]. Moreover, RI's determined with different assays in the past should be used with caution when applying cutoffs for folate deficiency on more recently obtained data, possibly resulting in misinterpretation of folate status [12]. The same holds true for assay recalibration by the manufacturer without changes being made to the core components of the assay, as has been performed by Roche in 2016.

Recently, RI estimation using the recalibrated Roche folate assay has been performed in Italy, the United Kingdom (UK) and Germany. The first study used healthy blood donors for which also the fruit and vegetable intake was recorded. Subject with abnormal haemoglobin, MCV levels or on folic acid supplements were excluded. As expected, the number of fruit or vegetable portions consumed significantly influenced folate values. Overall a RI (2.5–97.5th percentile) of 2.9–22.2 nmol/L (1.3–9.8 μg/L) was estimated [13], a considerably lower LRL than estimated in our cohort. As blood donors post-donation will have an increased erythropoiesis, the mean folate consumption will be higher in this group which could explain the reduced LRL obtained. As can be extrapolated from Fig. 2a, folate levels around 3 nmol/L (1.3 μg/L) coincide with elevated Hcy levels, indicating that this lower limit does not represent a sufficient folate level to maintain metabolic flux. The URL was however comparable to the one estimated by us using a gamma distribution. This probably indicates that values present in the right tail of our distribution represent individuals using vitamin supplements and could be considered as outliers. We decided to use the nonparametric RI with a higher upper limit as information on supplement use in our cohort was not available. The UK study used a large dataset of folate values measured in adults ordered by the GP. Herein, subject were excluded if Hb, MCV or vitamin B12 were outside the RI's. Also folate results outside the assay quantitative range were excluded. This study estimated the RI (2.5–97.5th percentile) at 5.4–39.7 nmol/L (3.4–17.5 μg/L), overall in good agreement with our results obtained by non-parametric estimation [14]. The authors decided to adhere to < 6.8 nmol/L (3 μg/L) as indicative for deficiency, in line with the British Society of Haematology guideline and comparable to the LRL estimated by us. A report from German researchers who used data mining of 2-year test results stored in the LIS of 57 medical centers estimated the folate RI (2.5–97.5th percentile) using the Bhattacharya method at 4.3–43.3 nmol/L (1.9–19.1 μg/L) [15]. Although all three mentioned publication established a LRL in their population, none validated their findings with observable effects on the metabolic pathway in which folate is required. In addition, the above mentioned studies did not use samples or data obtained from the general population.

In our study we tried to minimize possible effects from differences in folate intake and supplement use by collecting samples in the summer as well as in the winter season. Although both females and males showed a higher mean folate concentration in the winter cohort, a significant difference was only observed in males (Table 1). A possible explanation for the small increase in winter values could be related to increased use of multivitamin supplements during the winter season.

We decided to exclude data from individuals aged 5–20 years due to a significant lower mean folate level and due to contrasting results reported on folate levels between children and adults. For instance, a large German cohort study identified significant higher folate concentration in children (aged 6–17 years) than in adults [16]. On the other hand, a Canadian study reported lower folate levels in the 6–18 age group compared to adults [17]. These observed differences in folate concentration in children compared to adults between countries could be related to differences in local public health campaigns towards awareness related to folic acid requirements. Considering the inconsistency of published results on folate values in children in comparison with adults, we propose to apply the folate RI estimated for the age group 21–90 years independently of age possibly resulting in a small increase in children diagnosed with a folate deficiency.

Conflict of interest statement

All authors certify that they have no affiliations with or involvement in any organization with any financial interest in relation to the topic of the research described within this article.

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Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plabm.2019.e00127>.

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